Fatty Acids of Cows' Milk. B. Composition by Gas-Liquid Chromatography Aided by Other Methods of Fractionation[1]

1726

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A fresh sample of cows' milk was converted directly to the methyl esters by methanolysis and the esters were fractionated into eleven distilled fractions and an undistilled portion. The latter, which contained the bulk of the polyunsaturated and long chain esters, was further fractionated by adsorption chromatography on a silicic acid column. Each fraction from the distillation and adsorption chromatography was analyzed by gas-liquid chromatography on columns containing polyester stationary phases of different polarity. Twenty-seven minor components, including some not previously reported, were present each in less than 0.1%. The fatty acid distribution of the major components fell within the range generally reported.

PUBLISHED quantitative data on the fatty acids of cows' milk fat have generally been limited to less than half of the number of acids that have been reported in the fat owing to the difficulties of determining many of the minor components. Typical fatty acid analyses of various milk fats by methods other than gas-liquid chromatography (GLC) are given in a review by Jack and Smith (10). Many of the minor fatty acids with an estimation of the amounts found are reported by Shorland and Hansen (18). In the past few years, a number of papers have been published in which GLC has been employed to determine the fatty acids in various fractions of milk fat. The analysis of the total fatty acids of cows' milk fat or butterfat by GLC has also been attempted (2,4,11,14,15,17,19,20). The results were, in general, comparable to those determined by other methods, accounting for less than half of the total number of fatty acids. The difficulties of employing GLC to determine all acids present in milk fat were clearly recognized by Patton, *et al.* (15) who stated in part: "The problem of revealing the C_{19} to C_{26} fraction (components) seems to be particularly vexing, in that the quantity of the whole fraction probably is not more than 2% of the total, and the fraction emerges at a time in the chromatographing when the detection is poor due to spreading of components."

To achieve a more complete analysis of this extremely complex fat, distillation and column chromatography were employed to simplify the mixture before obtaining data by GLC. It was found advantageous to employ GLC columns which differed in polarity as a result of "aging" (13). Most fractions were hydrogenated as an aid in analysis. Ultraviolet and infrared spectrophotometry were also employed. The application of these supplementary techniques in conjunction with GLC for identification of at least 60 fatty acids of milk fat has been described by the authors (13). The same techniques were applied in the present work in which special attention was also given to estimation of percentage distribution of the individual fatty acids. Although milk fat from a different herd of cows was employed in this study, the same fatty acids were found present. The results bearing on the estimated percentage composition are presented.

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Experimental

Source of Milk. The milk was a composite sample of 10 qt from a herd of Ayrshire cows. The cows were fed a standard type grain-bran-molasses-mineral supplement in addition to timothy ad libitum, and 4 hr per day pasturage. The grain supplement was fed on the basis of the milk production of the cows (1 lb per 3 lb milk).

Extraction of Milk Fat. The freshly drawn milk was cooled and stored at 5C overnight. It was then centrifuged. The cream layer was removed, extracted several times in a separatory funnel with a mixture of 3 volumes of methanol to 2 volumes of ethyl ether, and finally with petroleum ether (bp 30–55C). The combined extracts were evaporated under reduced pressure (water pump) until free of solvent. Yield of fat, 330 g.

Conversion to Methyl Esters. To about 320 g of the milk fat was added 1 l. of 0.2 *N* methanol solution of sodium methoxide (made by adding freshly cut Na to methanol) and the mixture refluxed 40 min. The mixture was cooled, acidified, and poured into an equal volume of cold saturated salt solution, and extracted three times with petroleum ether. The combined extracts were washed with ice water until washings were neutral, and then dried over anhydrous sodium sulfate.

Distillation of Methyl Esters. The dry petroleum ether extract of methyl esters was distilled through a 30-in Vigreux column at atmospheric pressure to remove petroleum ether and some methyl butyrate. The amount of methyl butyrate contained in the petroleum ether forerun was determined independently by saponification number technique directly on the petroleum ether solution. The balance of the methyl esters was then fractionated under reduced pressures into eleven distilled fractions and an undistilled portion.

Column Chromatography. Silicic acid column chromatography was employed to further simplify the undistilled methyl ester fraction. The column and technique for operation under nitrogen were essentially the same as previously described (8,9,16). Eighty grams of a mixture of 80% silicic acid-20% filter aid were packed as a slurry in redistilled petroleum ether to a depth of 60 cm in a column 1.8 cm in diameter. Three grams of sample dissolved in a small amount of petroleum ether was introduced on the column and 4 successive fractions were eluted with petroleum ether. The next 3 fractions were eluted with increasing percentages of ethyl ether added to petroleum ether, depending on the progress of the fractionation. In all, 7 fractions were obtained.

TABLE I
Analysis of Fractions Obtained by Distillation of Milk Fat Methyl Esters

Fraction	Weight	I.V.	Conjugated[a]		Nonconjugated[a]			
			Diene	Triene	Diene	Triene	Tetra-ene	Penta-ene
	g.		%	%	%	%	%	%
Solvent[b]	7.8[b]	....	....	....	....	....	....	....
D 1	9.8	....	....	....	....	....	....	....
D 2	12.3	11.2	0.05	....	0.01	....	....	....
D 3	8.3	8.9	0.13	....	0.03	....	....	....
D 4	20.6	11.4	0.09	....	0.08	....	....	....
D 5	13.3	9.5	0.09	....	0.09	....	....	....
D 6	16.5	13.0	0.14	0.06	0.23	0.18	....	....
D 7	58.9	14.5	0.13	0.03	0.66	0.56	....	....
D 8	11.8	30.4	0.32	0.01	2.18	0.79	....	....
D 9	65.2	70.1	1.04	....	4.28	1.73	....	....
D 10	40.2	72.4	1.54	....	3.80	1.72	....	....
D 11	34.8	62.0	3.20	....	2.39	1.32	0.27	0.09
D 12-R	13.8	79.2	6.15	0.14	0.53	3.11	3.11	2.36
Summation[c]		40.6	1.10	0.02	1.90	1.01	0.17	0.11
Orig. Glyc.[a]		40.9	1.10	0.02	1.94	0.85	0.20	0.11
Orig. M.E.[a]		40.9	1.09	0.01	2.08	0.89	0.22	0.13
GLC[d]		....	0.72	0.01	2.17	0.63	0.19	0.10

[a] Analysis by spectrophotometric method (7).
[b] Solvent distilled from total methyl esters contained 7.8 g. of methyl butyrate.
[c] Calculated as per cent of total fatty acid esters from spectrophotometric analysis of D 1 to D 12-R.
[d] Calculated as per cent of total fatty acid esters from GLC analysis of all fractions.

Gas-Liquid Chromatography

Apparatus. The apparatus and thermal conductivity cell detector employed were the same as described previously (6,13).

Preparation of Stationary Phase. The ethylene glycol succinate polyester (EGS) was prepared by a modification of the general procedure for the preparation of various polyesters described by Carothers and Hill (1). One mole of dry glycol + 10% excess was heated together with 1 mole of acid or anhydride at 120–150C (depending on reactants) for 2 hr. Temperature was raised to 250C over the period of 1 hr. Pressure was then slowly reduced (15 min) to 2–3 mm and was maintained for an additional 5 min; heat was removed, mixture was allowed to cool, and vacuum was released. The polyester was ready for use without further treatment. A slow stream of nitrogen was passed through the reactants during the entire treatment. The final product (EGS) was a clear, colorless liquid which on cooling solidified to a hard, white solid.

Treatment of Solid Support. Chromosorb W was covered with concentrated HCl for 3 hr. at room temperature and the mixture stirred frequently. The acid was diluted with distilled water, decanted, and the solids transferred to a Buchner funnel and washed free of residual acid. The support was then treated in a similar manner with a mixture of 1 part NH_4OH + 3 parts water, washed free of base, and dried.

Column Packing. The support (treated Chromosorb W) was heated overnight at 145C just prior to coating with the stationary phase. The predetermined amount of polyester in methylene chloride was added to the warm support contained in a beaker in an amount sufficient to cover it completely. The mixture was stirred continuously with occasional heating in a hot water bath to keep the mixture warm until the odor of solvent was no longer detected. The column was packed with the coated support in the following manner: a compressed air vibrator was fastened to a clean and dry stainless steel tube closed at one end and clamped in a vertical position. While vibrating the column continuously, the coated support was added through a small funnel fastened to the column. When the column was full, it was vibrated 2 or 3 min longer and, if necessary, more packing was added. The ends were plugged with about ½ in of glass wool and then the column was coiled.

Columns. The column employed for obtaining the quantitative data was a coiled stainless steel tube 17 ft long and ¼ in OD (wall thickness—0.045 in). The column was packed with 35.3 g of acid and base washed 42 to 60 mesh Chromosorb coated with ethylene glycol succinate polyester. Weight of polyester was 7.06 g or 20% based on the total weight of packing. The column was heated to 203C for all runs except the fractions from the silicic acid fractionation where the temperature was increased to 235C. The pressure was maintained at 25 psig for the early distilled fractions and 35 psig for the later fractions. The flow of helium measured at the exit with a soap bubble column was 45 ml/min at the lower pressure and 70 ml/min at the higher pressure.

Calculation of Composition. The percentage fatty acid composition of each fraction was obtained by measuring the area under each peak on the chromatogram by either triangulation or with a planimeter and calculating the ratio of these areas to the sum of the areas under all of the component peaks. The order of agreement by this method to weight per cent of known mixtures is given in a previous publication (6).

TABLE II
Analysis by GLC of Distilled Fractions of Milk Fat Methyl Esters

Acid	D 1	D 2	D 3	D 4	D 5	Acid	D 6	D 7	D 8	D 9	D 10	D 11
	%	%	%	%	%		%	%	%	%	%	%
4:0[a]	9.78	.....	.....	.....	.....	12:0	0.07	.....	.....	.....	.....	.....
5:0	0.35	.....	.....	.....	.....	14:0	10.1	0.06	Trace	.....	.....	.....
6:0	74.2	0.48	.....	.....	.....	14:1	0.36	.....	.....	.....	.....	.....
7:0	0.56	0.11	.....	.....	.....	15:0brA[b]	1.83	.....	.....	.....	.....	.....
8:0	13.7	16.1	0.03	.....	.....	15:0brB[b]	3.91	0.07	.....	.....	.....	.....
9:0	0.04	0.61	.....	.....	.....	15:0	8.49	0.58	.....	.....	.....	.....
10:0	1.06	72.0	6.66	0.04	.....	15:1	0.78	0.02	.....	.....	.....	.....
10:1	0.14	6.26	0.73	.....	.....	16:0br	1.08	0.57	0.12	Trace	.....	.....
11:0	.....	0.42	0.68	.....	.....	16:0	60.9	85.7	64.7	7.70	0.11	.....
12:0	0.13	3.71	69.8	13.1	0.14	16:1	9.94	5.12	1.65	0.88	.....	.....
12:1A[b]	.....	0.20	1.60	0.36	.....	17:0brA[b]	0.32	0.75	2.07	0.45	0.16	.....
12:1B[b]	.....	0.08	1.63	0.37	.....	17:0brB[b]	0.34	0.44	1.36	0.37	0.13	.....
13:0br	.....	.....	0.69	0.39	.....	17:0	0.54	0.71	1.61	1.60	0.88	0.29
13:0	.....	0.04	0.54	0.67	.....	17:1	.....	0.31	1.21	0.67	0.20	.....
14:0br	.....	.....	0.30	1.08	0.38	18:0br	.....	.....	.....	Trace	Trace	.....
14:0	.....	.....	14.2	72.8	76.3	18:0	0.30	1.01	4.94	19.2	26.0	38.2
14:1	.....	.....	2.04	7.47	4.53	18:1	1.09	4.41	20.5	62.6	65.6	54.2
15:0brA[b]	.....	.....	0.21	0.72	2.10	18:2	.....	0.30	1.36	4.64	4.56	3.58
15:0brB[b]	.....	.....	.....	0.65	2.78	18:2 c,t conj.	.....	.....	0.14	0.67	0.90	1.73
15:0	.....	.....	0.35	0.97	3.83	18:3	.....	Trace	0.32	1.10	1.00	0.72
15:1	.....	.....	.....	.....	0.51	19:0	.....	.....	.....	0.21	0.34	0.72
16:0br	.....	.....	.....	.....	0.14	20:0	.....	.....	.....	.....	0.17	0.55
16:0	.....	.....	0.52	1.29	8.16							
16:1	.....	.....	.....	0.15	1.13							

[a] Additional methyl butyrate was found in solvent distilled from total esters. (See Table I.)
[b] A and B designate isomers.

Results and Discussion

In order to concentrate the components present in low concentrations, the methyl esters were fractionally distilled until most of the C_{18} esters, but little of the longer chain esters, were collected yielding 11 distilled and 1 undistilled fraction. The weights of the fractions, iodine numbers, and ultraviolet spectrophotometric analysis are shown in Table I. These data substantiate previous reports of the presence of conjugated dienoic acids and of nonconjugated acids with 2, 3, 4, and 5 double bonds. The summated results of analysis of these components by GLC and by the spectrophotometric method show a general order of agreement.

Each of the distilled fractions was first subjected to GLC and supplementary techniques as described (13) for identification of the components. After qualitative identifications of the acids were completed, gas-liquid chromatograms of each fraction were obtained for quantitative estimations. For this purpose, a chromatogram of each fraction was obtained on a new EGS column and then after the same column had aged, the fractions were again chromatographed. The effect of aging on the relative retention time of esters has been fully described by the authors (13). Many of the fractions were hydrogenated and rechromatographed to further elucidate the composition.

Table II shows the estimated percentage of the individual fatty acids in each of the distilled fractions. It was established by special treatment (13) that there are two positional isomers of dodecenoic acid, designated as 12:1A[3] and 12:1B, one having a terminal, the other an internal double bond. The presence of *cis* and *trans* isomers of 12:1 was also indicated. The difficulties of getting quantitative data are typified by the following example. Shown in Figure 1 are pertinent portions of chromatograms of a distilled fraction (D3) of milk fat esters. The three curves were obtained on the same 17 ft ethylene glycol succinate column. Figure 1a was obtained at 203C on a new column. On this chromatogram the 12:0br, 12:1A overlap and 12:1B and 13:0 are coincident. Figure 1b was obtained at 185C on the aged column and shows that 12:1A, 12:1B, and 13:0br are overlapped but 13:0 is separated. From this curve, the percentage of 13:0 can be determined and by difference from the values in Figure 1a, 12:1B is estimated. Figure 1c is a chromatogram of the fraction after hydrogenation. This curve was obtained on the aged column at 203C. The peak representing 13:0br is clearly shown and its percentage determined. Then from the total area percentage of 12:1A, 12:1B, and 13:0br on Figure 1b that due to 12:1B and 13:0br is subtracted and the per cent of 12:1A obtained by difference. The monoene containing an internal double bond was designated 12:1A, and the one containing a terminal double bond, 12:1B, since it has been demonstrated that esters with the double bonds closer to the carboxyl are less retentive than those farther from the carboxyl (5,12). Also shown on the curves is an example of a coincident peak of 10:1 and 11:0 (Fig. 1a) on the new column and its subsequent separation on the aged column (Fig. 1b). In this instance, the temperature was lowered to improve the separation but additional aging would have had the same effect. It was necessary to apply these techniques to many other fractions in order to estimate the percentage composition.

The undistilled fraction proved to be extremely complex. Qualitative techniques for identification showed that many of the peaks on the chromatogram represented 2 or more components, some of them esters of saturated and monoethenoid acids greater than 18 carbon atoms in chain length as well as polyunsaturated components. Hence, this material was further

[3] Shorthand designation as previously described (13).

TABLE III

Spectrophotometric Analysis of Conjugated Esters in Fractions Obtained by Silicic Acid Separation of D 12-R

Fraction	Weight	Diene	Triene
	g	%	%
C 1	0.69	.....	.....
C 2	7.13	0.38	.....
C 3	0.60	15.8	0.64
C 4	1.01	38.3	0.42
C 5	0.28	45.2	0.30
C 6	1.33[a]	16.2	0.79
C 7	2.80[b]	2.76	0.71

[a] Contained 0.13 g unsaponifiable matter.
[b] Contained 2.34 g principally unsaponifiable matter, monoglycerides, and free fatty acids.

TABLE IV

Analysis by GLC and Supplementary Techniques of Fractions Obtained by Silicic Acid Separation of 12 D-R

Acid	C 1	C 2	C 3	C 4	C 5	C 6	C 7
	%	%	%	%	%	%	%
18:0	14.2	54.2	2.14	0.28	0.11	.....	.....
18:1	.....	14.6	46.6	25.0	3.59	0.33	2.23
18:2	.....	.....	5.15	7.54	6.81	1.13	0.89
18:2 c,t conj.[a]	.....	.....	7.80	25.6	29.9	8.18	2.49
18:2 t,t conj.[a]	.....	.....	5.79	10.1	12.5	3.41	1.92
18:3	.....	.....	1.60	6.68	10.0	4.92	.....
18:3 conj.	.....	.....	0.64	0.42	0.30	0.79	.....
19:0	1.55	3.82	3.64	1.79	0.41	.....	.....
19:1	.....	2.12	5.28	1.88	0.43	.....	.....
20:0br	.....	Trace	.....	.....	.....	.....	.....
20:0	15.3	7.02	.....	.....	.....	.....	.....
20:1	.....	7.58	13.5	4.46	0.81	0.86	0.75
20:2	.....	.....	2.81	5.70	5.85	1.45	1.16
20:3	.....	.....	1.78	5.65	16.7	14.5	1.06
20:4	.....	.....	0.48	3.08	9.24	28.5	0.46
20:5	.....	.....	.....	.....	.....	8.61	0.27
21:0	3.88	1.10	0.88	0.33	1.00	.....	.....
21:1	.....	0.58	.....	0.18	0.51	0.66	.....
22:0	18.9	2.81	0.73	0.45	.....	.....	.....
22:1	.....	1.02	0.78	0.74	0.59	.....	.....
22:2	.....	.....	.....	.....	0.45	0.20	0.34
22:3	.....	.....	.....	.....	0.53	5.25	.....
22:4	.....	.....	.....	.....	.....	1.39	5.17
22:5	.....	.....	.....	.....	.....	8.24	2.95
23:0	7.86	0.75	.....	.....	.....	.....	.....
23:1	.....	1.35	0.36	Trace	.....	.....	.....
24:0	17.2	1.40	.....	.....	.....	.....	.....
24:1	.....	0.35	.....	.....	.....	.....	.....
25:0	2.46	.....	.....	.....	.....	.....	.....
26:0	18.6	1.37	.....	.....	.....	.....	.....
Unknown	.....	.....	.....	.....	0.32	1.69	.....
Nonmethyl ester	.....	.....	.....	.....	.....	10.0[b]	80.3[c]

[a] c,t = *cis-trans* and t,t = *trans-trans*.
[b] Unsaponifiable matter.
[c] Unsaponifiable matter, monoglycerides, and some free acids.

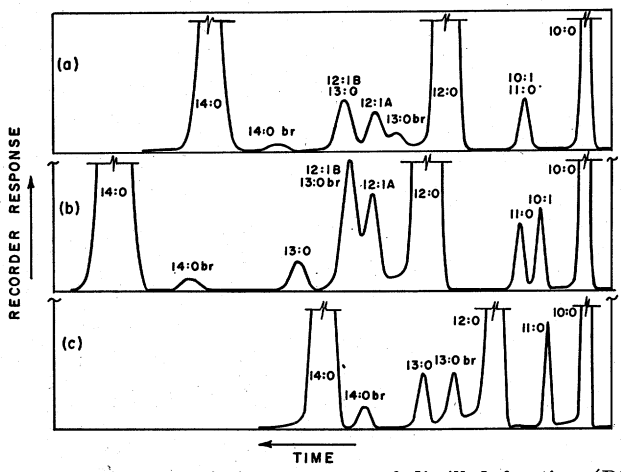

FIG. 1. Portion of chromatogram of distilled fraction (D3) of milk fat methyl esters. Column 17 ft x ¼ in, stationary phase, EGS polyester. (a) New column, temp 203C. (b) Aged column, temp 185C. (c) After hydrogenation, aged column, temp 203C.

separated on a silicic acid column into 7 fractions primarily on the basis of degree of unsaturation. Each of these was subjected to the same techniques of spectrophotometric and GLC analysis for identification and quantitative estimation as employed for the distilled ester fractions. The weights of the fractions and ultraviolet spectrophotometric analyses for conjugated diene and triene esters are shown in Table III. The values shown should be considered estimates since each conjugated isomer has a different absorptivity, and the percentage found would depend on the amount of each isomer present in the fraction. Further, the complete spectrophotometric analysis is not given since the method is not valid when large amounts of conjugated isomers are present, and also because the fractions from the silicic acid separation have a number of polyunsaturated esters for which absorptivities are not available. The complete fatty acid analyses by GLC are given in Table IV. Even though columns with different stationary phases and different temperatures were employed as described on these fractions from the silicic acid fractionation, separation was not achieved in two instances. In the first instance, nonconjugated 18:3 was not separated from conjugated *cis-trans* 18:2 although widening of the peak on the chromatogram could be seen. This problem was resolved by a determination of total conjugated diene ester by ultraviolet spectrophotometry and subtracting from it the amount of conjugated *trans-trans* 18:2 (calculated from its separate peak by GLC); the remainder was considered the amount of conjugated *cis-trans* 18:2. The amount of conjugated *cis-trans* 18:2 was in turn subtracted from the total amount found by GLC for the unresolved pair. The difference was the amount of 18:3 in the fraction. Infrared spectrophotometry was always employed to confirm the presence of *cis-trans* isomers. Similarly, nonconjugated 20:3 was not separable from conjugated 18:3. In this instance, the conjugated triene was determined by ultraviolet spectrophotometry and the amount of 20:3 was found by difference. Indication by GLC for presence of conjugated 18:3 was shown in a previous publication (13).

The individual fatty acids in the various fractions were summated and computed to the original total methyl esters of the milk fat to give the data in Tables V, VI, VII, and VIII. The presence of acids having *cis*, *trans* and terminal double bonds was confirmed by infrared spectrophotometry, but no attempt was made to give relative proportions of the geometrical isomers. Also, positional isomers of 16:1 and 18:1 have been reported. In this study, no attempt was made to confirm their presence. In all, 64 different fatty acids were accounted for, 27 of which were present in less than 0.1% concentration in the milk fat and would account for only 1% of the total fatty acids. With the exception of 17:1 recently reported (3), the homologous series of odd-carbon chain length monounsaturated acids 15:1 to 23:1 had not previously been reported in cows' milk fat.

TABLE V

Percentage of Saturated Acids in Total Acids of Milk Fat

Even		Odd	
Acid	%	Acid	%
4:0	2.79	5:0	0.01
6:0	2.34	7:0	0.02
8:0	1.06	9:0	0.03
10:0	3.04	11:0	0.03
12:0	2.87	13:0	0.06
14:0	8.94	15:0	0.79
16:0	23.8	17:0	0.70
18:0	13.2	19:0	0.27
20:0	0.28	21:0	0.04
22:0	0.11	23:0	0.03
24:0	0.07	25:0[a]	0.01
26:0	0.07	.....	.....

[a] Not previously reported.

TABLE VI

Percentage of Branched-Chain Acids[a] in Total Acids of Milk Fat

Even		Odd	
Acid	%	Acid	%
14:0br	0.10	13:0br	0.04
16:0br	0.17	15:0brA[b]	0.24
18:0br	Trace	15:0brB[b]	0.38
20:0br	Trace	17:0brA[b]	0.35
.....	.....	17:0brB[b]	0.25

[a] Number refers to total carbons in the compound; assignment more fully discussed in paper A (13).
[b] A and B designate isomers.

TABLE VII

Percentage of Monounsaturated Acids in Total Acids of Milk Fat

Even		Odd	
Acid	%	Acid	%
10:1[a]	0.27	15:1[d]	0.07
12:1[b]	0.14	17:1	0.27
14:1[b]	0.76	19:1[d]	0.06
16:1[c]	1.79	21:1[d]	0.02
18:1[c]	29.6	23:1[d]	0.03
20:1	0.22	.....	.....
22:1	0.03	.....	.....
24:1[d]	0.01	.....	.....

[a] Terminal double bond.
[b] Includes *cis*, *trans* and terminal double bond isomers.
[c] Includes *cis* and *trans* isomers.
[d] Not previously reported.

TABLE VIII

Percentage of Polyunsaturated Acids in Total Acids of Milk Fat

Dienes		Trienes	
Acid	%	Acid	%
18:2	2.11	18:3	0.50
18:2 c,t conj.[a]	0.63	18:3 conj.	0.01
18:2 t,t conj.[a]	0.09	20:3	0.11
20:2	0.05	22:3	0.02
22:2	0.01	.....	.....
Tetraenes		**Pentaenes**	
Acid	%	Acid	%
20:4	0.14	20:5	0.04
22:4	0.05	22:5	0.06

[a] c,t = *cis-trans* and *t,t* = *trans-trans*.

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